

Baseline Hepatitis C Virus (HCV) NS3 Polymorphisms and Their Impact on Treatment Response in Clinical Studies of the HCV NS3 Protease Inhibitor Faldaprevir

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A challenge to the treatment of chronic hepatitis C with direct-acting antivirals is the emergence of drug-resistant hepatitis C virus (HCV) variants. HCV with preexisting polymorphisms that are associated with resistance to NS3/4A protease inhibitors have been detected in patients with chronic hepatitis C. We performed a comprehensive pooled analysis from phase 1b and phase 2 clinical studies of the HCV protease inhibitor faldaprevir to assess the population frequency of baseline protease inhibitor resistance-associated NS3 polymorphisms and their impact on response to faldaprevir treatment. A total of 980 baseline NS3 sequences were obtained (543 genotype 1b and 437 genotype 1a sequences). Substitutions associated with faldaprevir resistance (at amino acid positions 155 and 168) were rare (<1% of sequences) and did not compromise treatment response: in a phase 2 study in treatment-naïve patients, six patients had faldaprevir resistance-associated polymorphisms at baseline, of whom five completed faldaprevir-based treatment and all five achieved a sustained virologic response 24 weeks after the end of treatment (SVR24). Among 13 clinically relevant amino acid positions associated with HCV protease resistance, the greatest heterogeneity was seen at NS3 codons 132 and 170 in genotype 1b, and the most common baseline substitution in genotype 1a was Q80K (99/437 [23%]). The presence of the Q80K variant did not reduce response rates to faldaprevir-based treatment. Across the three phase 2 studies, there was no significant difference in SVR24 rates between patients with genotype 1a Q80K HCV and those without Q80K HCV, whether treatment experienced (17% compared to 26%; $P = 0.47$) or treatment naïve (62% compared to 66%; $P = 0.72$).

The hepatitis C virus (HCV) NS3/4A protease is essential for viral replication and is a key target for the development of direct-acting antiviral agents for the treatment of chronic hepatitis C. Two HCV protease inhibitors, telaprevir and boceprevir, are approved for use in combination with pegylated interferon and ribavirin (PR) in patients infected with genotype 1 HCV (1). Two new HCV protease inhibitors have recently completed phase 3 evaluation and may become available in combination with PR in 2014 (faldaprevir [BI 201335] and simeprevir [TMC-435350]) (2–4). Various other classes of direct-acting antiviral agents are in late-stage clinical development and may provide additional treatment options (5). These new agents are likely to play an important role in combination therapy, both with and without interferon, for chronic hepatitis C in the near future (5).

One potential challenge to effective treatment with all direct-acting antiviral agents, including protease inhibitors, is the emergence of HCV drug-resistant variants. The high replication rate of HCV, coupled with the low fidelity and poor proofreading of its polymerase, generates a highly variable virus population, collectively called viral quasiespecies, and the creation of variants encoding amino acid substitutions that may result in reduced susceptibility to antiviral agents (6, 7). Under the selective pressure of antiviral treatment, resistant variants can rapidly become the majority population and lead to virologic failure. Indeed, in phase 1 studies with telaprevir, boceprevir, or faldaprevir monotherapy, virologic breakthrough was common, resulting in the necessity to combine these agents with PR to inhibit the emergence of resistant virus (6, 8–10). Protease inhibitor-resistant HCV variants selected

in vitro and *in vivo* have been shown to harbor mutations that encode amino acid substitutions in the NS3 protein (6, 11). Some NS3 amino acid substitutions reduce potency of most HCV protease inhibitors (e.g., at position 155). Others (at positions 36, 54, 55, and 170) are specifically associated with resistance to linear ketoamide protease inhibitors that form a reversible, covalent bond with the catalytic serine of NS3/4A protease, such as boceprevir and telaprevir, and substitutions at NS3 168 are generally specific to noncovalent protease inhibitors, such as the linear tripeptide faldaprevir and the macrocyclic compounds simeprevir, asunaprevir, and vaniprevir. Some substitutions are associated with subsets of protease inhibitors, such as substitutions at positions 80 and 122, which are associated with resistance to simeprevir (4, 11).

Several groups have reported the detection of HCV variants with natural NS3/4A polymorphisms that are associated with protease inhibitor resistance in PR treatment-naïve and/or protease

Received 23 September 2013 Returned for modification 10 October 2013

Accepted 6 November 2013

Published ahead of print 11 November 2013

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doi:10.1128/AAC.01976-13

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TABLE 1 Summary of clinically relevant NS3 amino acid changes associated with reduced susceptibility to HCV protease inhibitors (11)

Drug	Substitution(s) ^a detected in patients with virologic failure at NS3 amino acid position:											
	36	43	54	55	80	122	132	155	156	158	168	170 175
Faldaprevir								<u>K</u> , Q	T		<u>V</u> , E, A, N, T	
Simeprevir					K, R	A, G, R		<u>K</u> , Q			<u>V</u> , E, A, H	T
Vaniprevir		S						<u>K</u> , G, T			<u>V</u> , Y, G, A	
Boceprevir	<u>M</u> , A		<u>A</u> , <u>S</u> , C, G	<u>A</u>				<u>K</u> , T	<u>S</u> , T, V	I	N	<u>A</u> , T L
Telaprevir	<u>M</u> , <u>A</u> , L, G, I		<u>A</u> , <u>S</u>				V	<u>K</u> , T, G, M	<u>S</u> , T, V, F, N		N	

^a Underlined letters indicate variants reported in $\geq 10\%$ of patients failing treatment in phase 2 or phase 3 studies (others are reported in $<10\%$ of patients failing treatment in phase 2 or phase 3 studies).

inhibitor-naïve patients, in particular polymorphisms at NS3 codon 80 (12–20). It is not clear how these baseline polymorphisms impact response to treatment. Clinical study data suggest that response to simeprevir plus PR may be reduced among patients infected with HCV genotype 1a with baseline Q80K substitutions (21). With several new direct-acting antivirals for the treatment of HCV approaching approval, it will be important to optimize treatment to ensure that all patients have the best chance of success. Therefore, a clear understanding will be needed of how the presence of resistance-associated variants at baseline influences treatment response.

Faldaprevir is a reversible inhibitor of HCV NS3/4A protease that has been investigated in phase 1b, phase 2, and phase 3 clinical studies for the treatment of patients infected with HCV genotype 1 (2, 3, 22–24). In phase 1b clinical studies, faldaprevir was well tolerated and induced a rapid and steep dose-related virologic response within 2 to 4 days of initiation of monotherapy or combination therapy with PR (22). In a phase 2 clinical study, faldaprevir in combination with PR achieved SVR in up to 84% of treatment-naïve patients infected with HCV genotype 1 (2). We performed a comprehensive analysis of pooled data from phase 1b and phase 2 clinical studies with faldaprevir to assess the frequency of baseline NS3/4A polymorphisms associated with protease inhibitor resistance and the impact of these polymorphisms on response to faldaprevir-based treatment.

MATERIALS AND METHODS

Patients and treatment. The analysis was performed on samples derived from patients infected with HCV genotype 1 enrolled in two phase 1b clinical studies and three phase 2 clinical studies, which have been described previously (2, 3, 22–24).

In the first phase 1b study (1220.2, NCT00793793), performed in Europe and the United States, treatment-naïve patients ($n = 34$) received placebo or faldaprevir monotherapy (20 to 240 mg once daily [QD]) for 14 days followed by faldaprevir or placebo plus PR for 14 days (10, 22). Patients previously treated with PR ($n = 62$) received faldaprevir (48 to 240 mg QD or 240 mg twice daily [BID]) and PR for 4 weeks. In the second phase 1b study (1220.14, NCT00947349), Japanese treatment-naïve ($n = 16$) and treatment-experienced ($n = 6$) patients received faldaprevir (120 or 240 mg QD) or placebo for 4 weeks followed by PR for 44 weeks (23).

Phase 2 studies were performed in Europe, the United States, Canada, South America, and Australia. The SILEN-C1 (NCT00774397) phase 2 study included treatment-naïve patients ($n = 429$) who received 120 mg faldaprevir QD and PR up to week 24 with a 3-day lead-in period of PR alone, or 240 mg faldaprevir QD and PR up to week 24 with and without the lead-in period, or placebo and PR (2). In the SILEN-C3 (NCT00984620) phase 2 study, treatment-naïve patients ($n = 159$) received 120 mg faldaprevir QD and PR for 12 or 24 weeks with a 3-day lead-in period of PR alone (24). In the SILEN-C2

(NCT00774397) phase 2 study, treatment-experienced patients ($n = 288$) received 240 mg faldaprevir BID and PR with a 3-day lead-in period of PR alone, or 240 mg QD with and without lead-in, for a treatment duration of 24 weeks (3). In all phase 2 studies, total treatment duration of PR was 24 or 48 weeks.

All patients provided written informed consent, and all studies were performed in accordance with the Declaration of Helsinki (1996 version), the International Committee on Harmonization (ICH) Harmonized Tripartite Guideline for Good Clinical Practice (GCP), and applicable local regulatory requirements.

Amplification and sequencing of HCV NS3/4A. Viral RNA was isolated from baseline plasma samples using the QiaAmp viral RNA extraction kit (Qiagen, Hilden, Germany). The HCV RNA level of all baseline samples was $>1,000$ IU/ml, the lower limit of amplification. Reverse transcription and PCR (Superscript II one-step reverse transcription [RT]-PCR system with Platinum Taq; Invitrogen, California) was used to amplify a 2.4-kbp DNA fragment spanning the complete NS3/4A region from nucleotide positions 3276 in NS2 and 5650 in NS4B (forward RT-PCR primer, 5'-ATGGAGACCAAGCTCATCACCTGGGGGGCAGACA CC-3'; reverse RT-PCR primer, 5'-CTGATGAAATTCACATGTGCTT CGCCCAGAAAGG-3'). Nucleotide positions are based on the full genome sequence of H77 and Con-1 (accession numbers below). A second-round, seminested PCR was performed using the high-fidelity KOD Hot Start DNA polymerase kit (Novagen; Merck, Darmstadt, Germany) to generate a 2.3-kbp fragment of the complete NS3/4A region from nucleotide positions 3276 in NS2 to 5542 in NS4B (forward primer, same as that used for RT-PCR; reverse PCR primer, 5'-CCGAGCGCCTTCTGCTTGAAGTGC TCGGCGAG-3'). Population sequencing of the 2.3-kbp amplification product was performed with bidirectional primers to achieve $\geq 90\%$ double-strand coverage for the NS3/4A region using BigDye Terminator version 3.1 and the ABI Prism 3730XL Genetic Analyzer (Applied Biosystems, California). The NS3/4A nucleotide sequences (2,055 bp) were analyzed with SeqScape version 2.5 (Applied Biosystems, California). Forward sequencing primers included the forward RT-PCR primer and primers spanning NS3 nucleotide positions 3813 to 3831, 4278 to 4297, and 4467 to 4484. Reverse sequencing primers included the reverse PCR primer and primers spanning NS3 nucleotide positions 3736 to 3753, 4543 to 4562, 4713 to 4732, 5017 to 5034, and 5101 to 5127. For samples that could not be amplified by the standard amplification and sequencing primers, degenerate GT1 or alternative subtype-specific primers were used. The fidelity of the reverse transcription and PCR process was previously verified through clonal sequence analysis (~ 90 clones) of pre-defined mixtures of benchmark site-directed mutants to ensure that the amplifications were within the linear range and sensitive to detection of minor variants (10).

Mixed nucleotide codons encoding mixed amino acids were assigned when they were represented by at least 10% of the surface area of the major peak in the DNA electropherogram. To identify nucleotide and amino acid changes, sequences were compared to reference sequences appropriate for the genotype 1 subtype: H77 (GenBank accession number AF009606) for subtype 1a and Con-1 (GenBank accession number AJ238799) for subtype 1b. Full-length NS3/4A nucleotide population se-

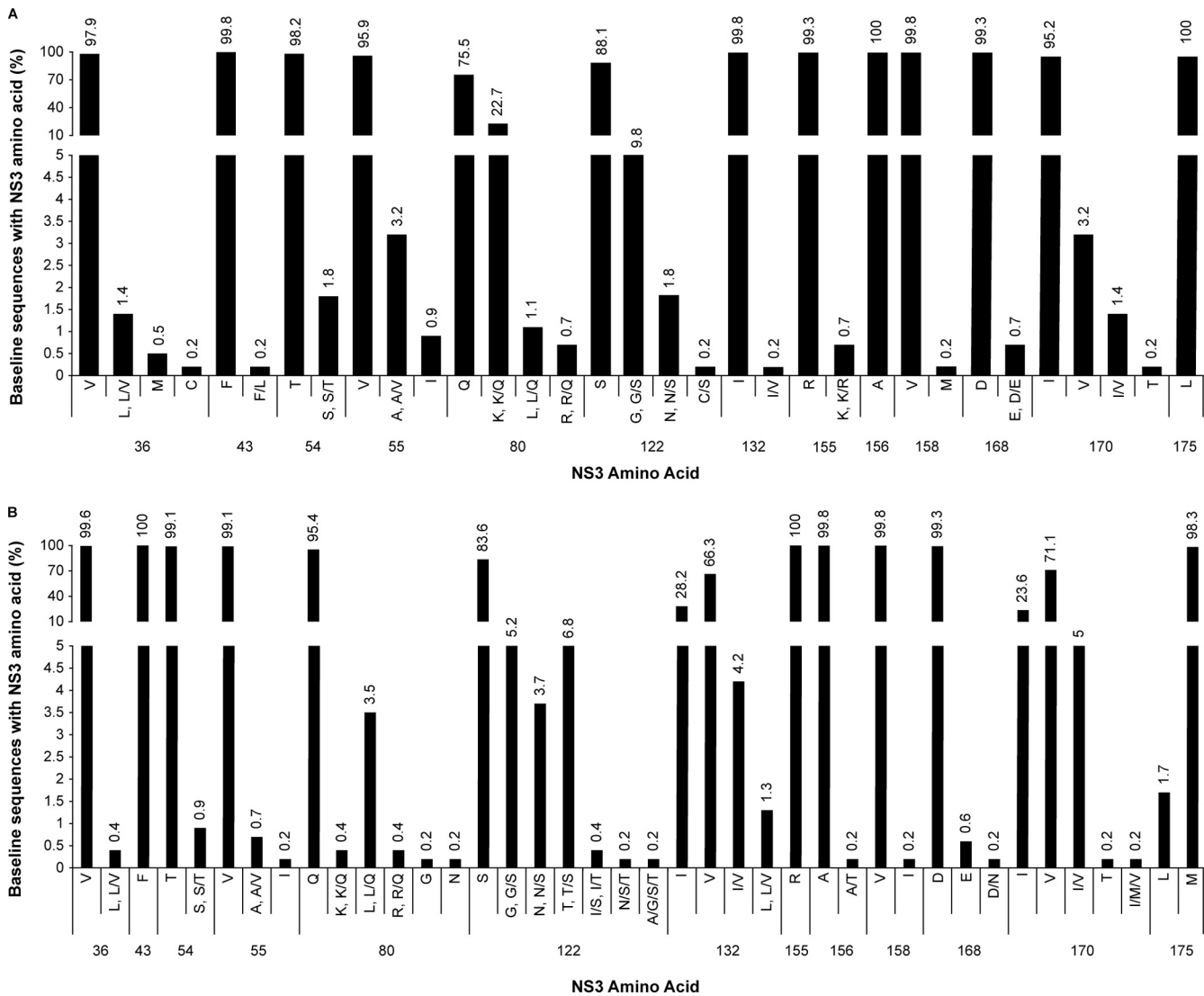


FIG 1 Baseline frequency of polymorphisms at amino acid positions associated with NS3 protease inhibitor resistance among patients infected with HCV genotype 1a (A; $n = 437$) or HCV genotype 1b (B; $n = 543$) from phase 1b and phase 2 clinical studies of faldaprevir. Amino acid changes were identified by population sequencing and with respect to the reference amino acid for the genotype 1 subtype: H77 ([AF009606](#)) for subtype 1a and Con-1 ([AJ238799](#)) for subtype 1b. Observed polymorphisms at each amino acid position are indicated on the x axis.

sequences were aligned with the subtype 1a and subtype 1b references in the AlignX module of the VectorNTI Advance 10 software (Invitrogen), and HCV subtype was assigned using the guide tree of the alignment.

Site-directed mutagenesis and phenotypic drug susceptibility assay. Changes in HCV protease inhibitor susceptibility conferred by single amino acid variants generated by site-directed mutagenesis (QuikChange Lightning site-directed mutagenesis; Stratagene, California) were evaluated by conducting *in vitro* transient HCV replication assays. The genotype 1b construct is a bicistronic HCV subgenomic replicon comprising a luciferase reporter gene and an adapted con-1 NS3 to NS5B region (10). The genotype 1a replicon consisted of the same genotype 1b backbone but encoded a genotype 1a NS3 protease sequence (codons 11 to 218, inclusive). Linearized plasmid DNA was used to generate HCV subgenomic replicon RNA transcripts (T7 Ribomax kit; Promega, Wisconsin). HCV RNAs were electroporated into Huh-7.5 cells and seeded at a density of 12,500 cells per well of a 96-well plate, and luciferase activity (Promega, Wisconsin) was measured 96 h later as a marker for HCV RNA replication. To assess susceptibility to HCV inhibitors, serial dilutions

of inhibitors were added to the cells 24 h postelectroporation, and the concentration giving 50% inhibition of HCV RNA replication (EC_{50}) was determined using a four-parameter logistic (Hill equation). Protease inhibitors for use in these assays were synthesized by Boehringer Ingelheim, Canada Ltd., R&D.

Analyses. Baseline NS3/4A population sequences from genotype 1a and genotype 1b isolates from the five clinical trials were pooled. The rare HCV non-1 genotypes and other genotype 1 isolates (non-1a, non-1b) were sequenced but were excluded from these analyses. Changes with respect to HCV subtype references were noted, and the frequency of polymorphisms was calculated for 13 amino acid residues that are associated with resistance to NS3/4A protease inhibitors in treated patients (resistance-associated amino acid sites). Clinically relevant NS3 amino acid sites were defined according to tables published by the HCV Phenotype Working Group of the Forum for Collaborative HIV Research and are summarized in [Table 1](#) (11). To ensure that the NS3 polymorphisms observed in our clinical study population were representative of the natural genetic diversity of HCV, we com-

TABLE 2 Summary of reported baseline frequency of clinically relevant NS3 amino acid changes in patients chronically infected with HCV genotype 1a and naive to HCV protease inhibitors^f

Study characteristics	Reported amino acid substitution(s) (%) ^a at NS3 amino acid position:												
	V36	F43	T54	V55	Q80	S122	I132	R155	A156	V158	D168	I170	L177
EU HCV and Los Alamos 2008 (25), <i>n</i> = 131	M (<1)	—	S (<1)	NA	K (34), R (<1)	NA	NA	K (<1)	—	NA	E (<1)	—	NA
Gilead Science and Los Alamos 2010 (12), ^b <i>n</i> = 268	NA	NA	NA	NA	K (47)	NA	NA	NA	NA	NA	NA	NA	NA
Los Alamos 2013 (18), <i>n</i> = 680	L (<1), M (<1)	S (<1)	S (1)	NA	K (37), R (<1)	NA	NA	G (<1), K (<1)	—	NA	E (<1), G (<1)	—	NA
GenBank analysis 2012 (15), <i>n</i> = 621	L (<1)	—	—	A (2)	K (42)	NA	NA	—	—	NA	E (<1)	—	—
French cohort (26), <i>n</i> = 46	NA	NA	NA	NA	K (7)	G (9)	V (2)	NA	NA	NA	NA	NA	NA
Italian cohorts (13, 14), ^c <i>n</i> = 98	L (6)	—	S (4)	A/I (3)	K (14)	NA	NA	—	—	—	—	—	—
Swedish cohort (17), <i>n</i> = 53	L (4)	NA	A (2), S (8)	A (8), I (6)	K (6), R (4)	—	NA	NA	NA	NA	—	NA	NA
Brazilian cohort (19), ^d <i>n</i> = 37	—	—	A (3), S (3)	A (3)	K (3), R (3)	G (5)	—	K (3)	T (5)	—	G (3)	—	—
Scottish cohort (20), ^e <i>n</i> = 38	M (5)	—	S (3)	A (3)	K (16)	NA	NA	—	—	NA	A (3)	—	NA
Telaprevir clinical studies (16), <i>n</i> = 2,111	I/M (<1), L (2)	—	A (<1), S (3)	A (3), I (2)	K (38), R (<1)	NA	V (<1)	K (<1)	—	—	E (<1)	T (<1)	—
Faldaprevir clinical studies (present analysis), <i>n</i> = 437	L (1), M (<1)	—	S (2)	A (3), I (<1)	K (23), R (<1)	G (10)	V (<1)	K (<1)	—	—	E (<1)	T (<1)	—

^a In populations of protease inhibitor-naïve patients infected with HCV genotype 1a. Population-based sequencing for NS3 sites with no variant listed, the wild-type amino acid, or amino acids not associated with PI resistance were reported (—) or data were not available (NA).

^b Only positions 54 and 80 were reported.

^c Two studies carried out in Italy and reporting similar results were pooled. Included 55 HIV/HCV-coinfected patients.

^d Genotype not confirmed. Based on the prevalent genotype in the region of study.

^e HIV/HCV-coinfected patients.

^f In the present analysis and in previously published studies.

pared our results with NS3 sequences derived from treatment-naïve patients and available in the public domain (Los Alamos Database [http://hcv.lanl.gov/content/index, accessed January 2013] and GenBank database [http://www.ncbi.nlm.nih.gov/GenBank/index.html; accession numbers KC123434 to KC127656]), including 3,571 genotype 1a NS3 sequences and 2,955 genotype 1b NS3 sequences.

The relationship between baseline NS3 polymorphisms and SVR was investigated for patients treated with faldaprevir and PR in the phase 2 clinical studies. Fisher's test was used to compare SVR rates among patients with and without specific baseline polymorphisms.

RESULTS

NS3 protease domain polymorphisms detected at baseline. A total of 980 baseline NS3/4A population sequences from genotype 1a and genotype 1b isolates were obtained: 118 from patients in phase 1b studies and 862 from patients in phase 2 studies (see Materials and Methods for regions of patient recruitment). Among these were 543 genotype 1b sequences and 437 genotype 1a sequences. Figure 1 summarizes the frequency of amino acids identified at 13 clinically relevant amino acid positions in the NS3 protease domain that are associated with NS3 protease inhibitor resistance. Among these 13 amino acid positions, the greatest heterogeneity in genotype 1a sequences was detected at NS3 codon 80, with 332/437 (76%) sequences coding for the reference amino acid (wild type), glutamine (Q). The most common amino acid substitution seen at genotype 1a NS3 codon 80 was Q80K (99/437 [23%]) (Fig. 1 and Tables 1 and 2). Few polymorphisms were detected at codon 80 in genotype 1b sequences, the most common substitution being Q80L (19/543 [3.5%]). Among the 13 amino acid positions, the greatest heterogeneity in genotype 1b sequences was detected at NS3 codon 132, with 360/543 (66%) sequences coding for the wild-type valine (V), and NS3 codon 170, with 386/543 (71%) sequences coding for the wild-type V. Sequences at position 170 coded almost exclusively for isoleucine (I; wild type for genotype 1a) or V (wild type for genotype 1b) in both subtypes; 170T (an amino acid position associated with resistance to boceprevir) was a rare polymorphism detected in only 1/437 genotype 1a and 1/543 genotype 1b sequences. Substitutions at amino acid positions 155 and 168 (associated with faldaprevir resistance) were also rare and were detected in <1% of baseline population sequences (R155K, 3/437 genotype 1a; D168E, 3/437 genotype 1a; D168E, 3/543 genotype 1b; D168N, 1/543 genotype 1b).

Amino acid conservation and variants noted in the Los Alamos and GenBank public databases were generally consistent with the diversity of NS3 baseline sequences obtained from clinical isolates in faldaprevir clinical studies (data not shown). This demonstrates that there was adequate representation of diverse HCV genotype 1a and genotype 1b isolates in our study population.

Impact of baseline amino acid substitutions on *in vitro* susceptibility to faldaprevir. A previous comprehensive phenotypic analysis of NS3 proteases that were derived from baseline clinical isolates showed that only Q80K and V170T substitutions conferred notable changes in faldaprevir *in vitro* susceptibility (25). The impact of the Q80K and V170T substitutions on susceptibility to protease inhibitors was further evaluated using site-directed mutagenesis to introduce mutations encoding these individual substitutions into genotype 1b subgenomic replicons (V170T) or a chimeric genotype 1b subgenomic replicon-encoding genotype 1a NS3 protease (Q80K). The results are summarized in Fig. 2. Genotype 1a NS3 Q80K conferred only a 3.2-fold reduction in

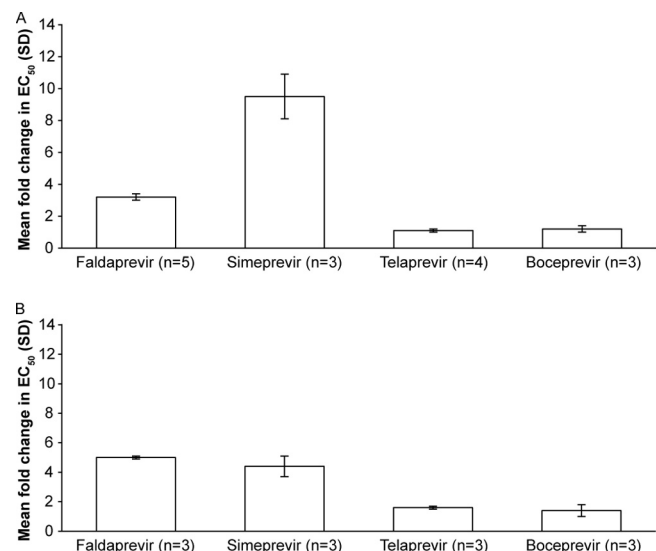


FIG 2 Susceptibility to NS3/4A protease inhibitors of HCV replicons modified by site-directed mutagenesis to encode Q80K in genotype 1a NS3 (A) or V170T in genotype 1b NS3 (B). Results are expressed as fold change in EC₅₀ relative to the wild-type control. Bars show the means (\pm standard deviations) from at least three experiments (n = number of experiments).

susceptibility to faldaprevir relative to the wild type. The impact of this substitution on susceptibility to simeprevir was greater than that observed for faldaprevir, with a 9.5-fold reduction in susceptibility with genotype 1a Q80K (Fig. 2A). V170T in genotype 1b

resulted in a 5.0-fold reduction in susceptibility to faldaprevir and a 4.4-fold reduction in susceptibility to simeprevir (Fig. 2B). Q80K or V170T site-directed mutants had no apparent impact on *in vitro* susceptibility to telaprevir or boceprevir and had replication capacities similar to those of wild-type replicons; the mean (\pm standard deviation) replication capacity was 94% (\pm 15%) for 1a Q80K (n = 8 experiments) and 93% (\pm 32%) for 1b V170T (n = 4 experiments).

Effect of key baseline NS3 polymorphisms on faldaprevir antiviral response. In the SILEN-C1 phase 2 study (treatment-naïve patients), genotype 1a HCV with the R155K substitution was detected in two faldaprevir-treated patients at baseline, and substitutions at amino acid position 168 were detected in four faldaprevir-treated patients at baseline (D168E in two genotype 1a and one genotype 1b and D168N in one genotype 1b). Five of these patients achieved an undetectable viral load during treatment (HCV RNA of <1 log₁₀ IU/ml) and achieved SVR24 posttreatment (Fig. 3); one patient with the genotype 1a D168E substitution experienced a viral load decline but discontinued prematurely at week 4 and did not achieve SVR (data not shown). One patient with the R155K baseline polymorphism, treated with 240 mg faldaprevir QD and PR, achieved SVR despite a slower viral load decline. NS3 I170T was detected together with R155(K/R) in a second patient, treated with 240 mg faldaprevir QD and PR, who achieved SVR. The only other (I/V)170T polymorphism detected was in one genotype 1b patient in the phase 1b study 1220.2 (in the 20-mg QD low-dose faldaprevir arm) who demonstrated a virologic response to treatment (10).

In the small phase 1b study, baseline genotype 1a Q80K was de-

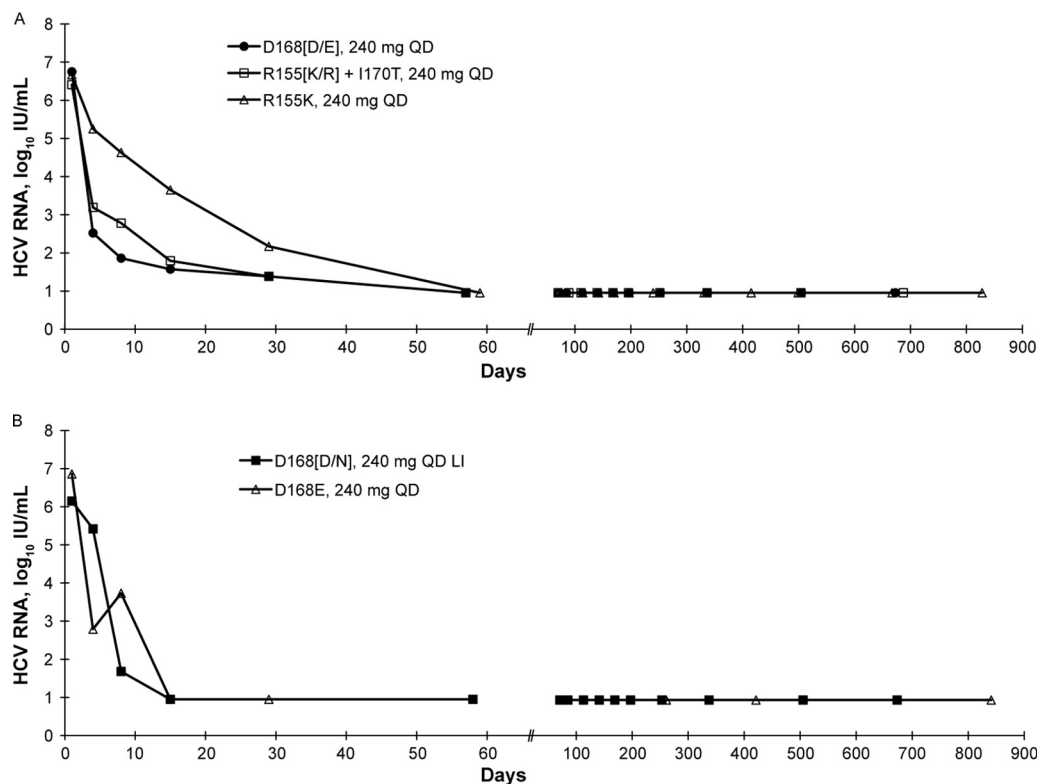


FIG 3 Reduction in viral load during faldaprevir and PR treatment in patients with baseline R155 or D168 variants in the SILEN-C1 phase 2 study. Genotype 1a-infected (A) and genotype 1b-infected (B) patients. Faldaprevir dose for each patient is shown. PR, pegylated interferon-ribavirin; QD, once daily; LI, 3-day lead-in period of PR.

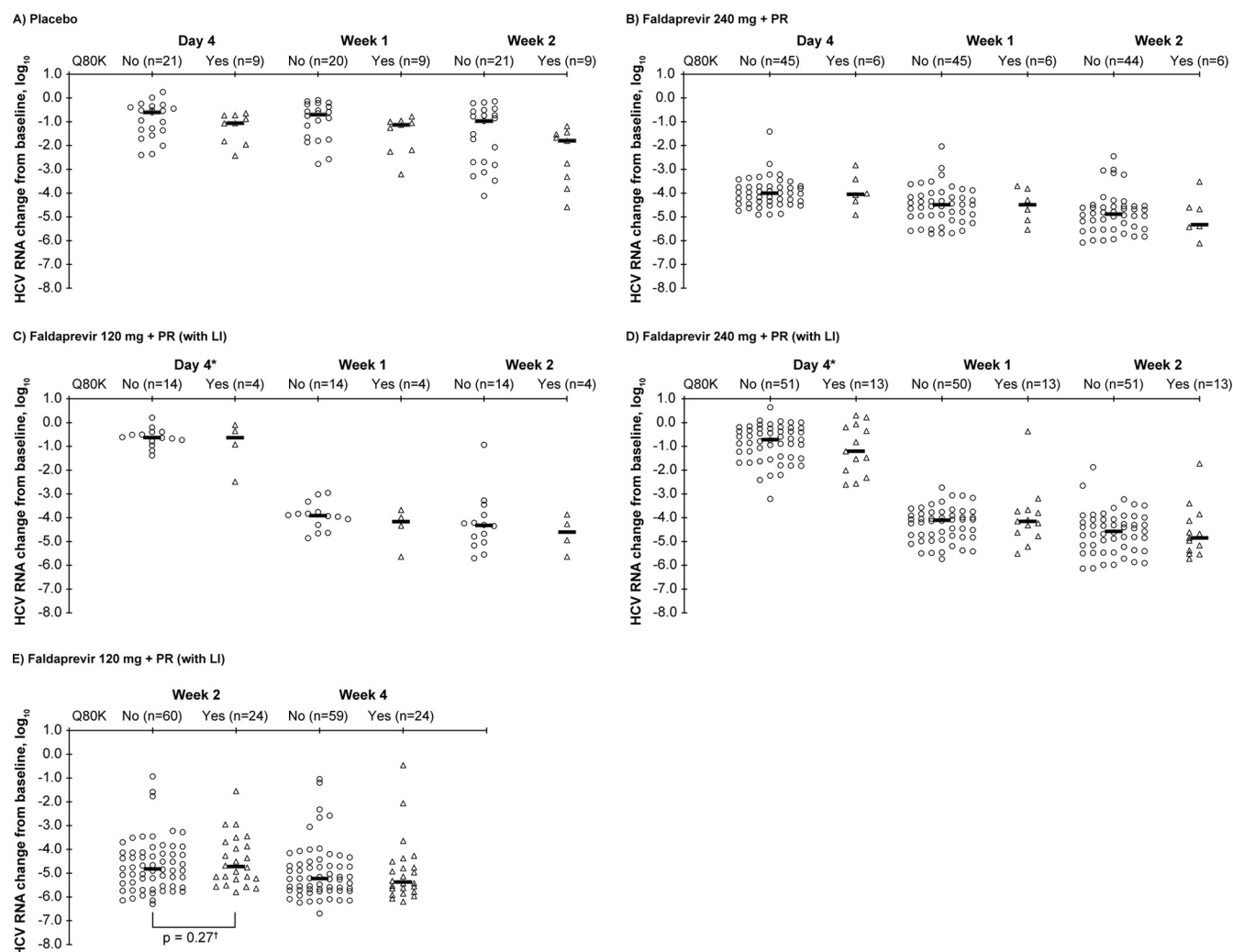


FIG 4 Viral load decline in treatment-naïve patients infected with genotype 1a HCV with (triangles) or without (circles) baseline NS3 Q80K in phase 2 studies. (A) Placebo (SILEN-C1); (B) 240 mg faldaprevir QD and PR (SILEN-C1); (C) 120 mg faldaprevir QD and PR with LI (SILEN-C1); (D) 240 mg faldaprevir QD and PR with LI (SILEN-C1); (E) 120 mg faldaprevir QD and PR with LI (SILEN-C1 and SILEN-C3). Each symbol represents one patient, and the horizontal lines show the median for all patients. Q80K analysis includes predominantly Q80K and one Q80(K/Q) in panel C and two Q80(K/Q) in panel E. “Without Q80K” analysis includes predominantly wild-type Q80 and one Q80R and one Q80(Q/R) in panel D and one Q80L in panel E. Visit schedules in SILEN-C1 and SILEN-C3 first overlapped at week 2, as shown in panel E, and data were pooled for all patients treated with LI and 120 mg faldaprevir QD and PR. *, day 4 preceded by a 3-day lead-in period of PR treatment only; †, Wilcoxon rank sum test (carried out on the largest group at the lowest faldaprevir dose at the earliest time point); LI, lead-in; PR, pegylated interferon-ribavirin; QD, once daily.

tected in 15/52 (29%) patients; and at faldaprevir doses of 48 mg, 120 mg, and 240 mg QD, the initial viral load decline did not differ between patients with and without the Q80K polymorphism (10). The impact of the Q80K baseline polymorphism on the antiviral response to faldaprevir was examined in a larger number of patients in the SILEN-C1 and SILEN-C3 phase 2 studies. The median initial viral load decline in response to faldaprevir treatment in treatment-naïve patients in the SILEN-C1 and SILEN-C3 studies did not differ between patients with and without the Q80K polymorphism at baseline at either 120 mg QD or 240 mg QD doses of faldaprevir (Fig. 4). Across all three phase 2 studies, genotype 1a HCV with a Q80K substitution was detected at baseline in 45 treatment-naïve patients and 29 treatment-experienced patients; treatment groups were pooled to obtain a larger sample size of Q80K to evaluate. Furthermore, the analysis was pooled across the various dose regimens since there was

no correlation between dose or duration of treatment and response to treatment in phase 2 studies (2, 3). In treatment-naïve patients, 65.8% (106/161) of patients without Q80K HCV achieved SVR24 compared to 62.2% (28/45) with Q80K ($P = 0.72$, Fig. 5); in treatment-experienced patients, 26.3% (31/118) of patients without Q80K HCV achieved SVR24 compared to 17.2% (5/29) with Q80K ($P = 0.47$; Fig. 5). Thus, no significant difference was observed in SVR24 rates between patients, either treatment naïve or treatment experienced, with the Q80K polymorphism and those without.

DISCUSSION

The pooled analysis of phase 1b and phase 2 studies of faldaprevir provided a large number of patient-derived HCV NS3/4A sequences for the assessment of baseline polymorphisms. Baseline polymorphisms were observed at only a limited number of

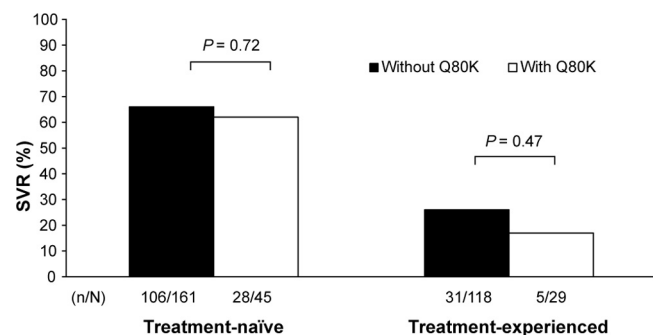


FIG 5 Virologic response to faldaprevir and PR in patients infected with genotype 1a HCV with or without the NS3 Q80K polymorphism at baseline (pooled phase 2 studies). Q80(K/Q) mixtures detected in two treatment-naïve patients and one treatment-experienced patient are included in the Q80K columns. Statistical analysis (Fisher's test) compared samples with Q80K to those without Q80K. Analysis of treatment-naïve patients includes the groups of 120 mg faldaprevir QD and LI (12 or 24 weeks) and 240 mg faldaprevir QD with or without LI treatment. Analysis of treatment-experienced patients includes the groups of 240 mg faldaprevir QD with or with LI and 240 mg faldaprevir BID and LI treatment. LI, lead-in; PR, pegylated interferon-ribavirin; SVR, sustained virologic response 24 weeks after the end of all therapy.

amino acid positions that are associated with protease inhibitor resistance. Variants specifically associated with faldaprevir resistance (at codon 155, 156, or 168) were rare in both genotype 1a and genotype 1b sequences. This is consistent with previous studies assessing the frequency of polymorphisms in HCV NS3, which have reported faldaprevir resistance-associated mutations in 0 to 3% of genotype 1a or 1b sequences (12–20, 25, 26). The small number of patients who were infected with variants encoding faldaprevir resistance-associated mutations responded to treatment with faldaprevir and PR in phase 2 studies.

The Q80K substitution, which confers resistance to the macrocyclic protease inhibitor simeprevir (4), was a common polymorphism observed among genotype 1a sequences, occurring in 23% (99/437) of patients. This observation is consistent with results published by several other groups, as summarized in Table 2. In patients infected with HCV genotype 1a and naïve to HCV direct-acting antivirals, the Q80K substitution has been reported in 3 to 47% of patients (12–20, 25, 26). The variation in the frequency of Q80K detection in different studies appears to be influenced by the geographical origin of HCV genotype 1a isolates and likely reflects geographical differences in the distribution of genotype 1a clades. Two lineages of genotype 1a have been reported (14, 27), with clade 1 having a higher prevalence (72.7%) in sequences obtained from the Los Alamos HCV database (in which the majority of isolates are from the United States) relative to sequences from an Italian cohort (47.3% prevalence of clade 1) (28), thus highlighting a difference between North American and European populations of HCV genotype 1a isolates. Furthermore, the Q80K variant was more frequent in clade 1 than clade 2 (48.9% compared to 0%, respectively; $n = 293$ sequences) (28).

In drug sensitivity assays, a genotype 1a NS3 Q80K site-directed mutant showed a small reduction in susceptibility to faldaprevir (3-fold mean change in EC_{50} compared with that of the wild type). This confirms previously published observations from an analysis of HCV chimeric subgenomic replicons encoding NS3 proteases derived from patients in the phase 1b multiple-rising-dose study 1220.2; ge-

notype 1a baseline clinical isolates with the Q80K polymorphism had a 2-fold reduction in susceptibility to faldaprevir compared with that of isolates without Q80K (10). This small reduction in susceptibility to faldaprevir would not be expected to compromise the virologic response to faldaprevir treatment since the faldaprevir EC_{50} for Q80K variants remains more than 100-fold below faldaprevir trough levels achieved in humans treated with faldaprevir at 120 mg daily (10, 22). Consistent with these observations, the initial viral load decline in response to faldaprevir in treatment-naïve patients included in phase 2 studies (SILEN-C1 and SILEN-C3) were comparable between patients with or without Q80K variants. In addition, among patients infected with HCV genotype 1a across three phase 2 studies in treatment-naïve and treatment-experienced patients, there was no significant difference in SVR rates after treatment with faldaprevir and PR between patients with and without Q80K variants at baseline. Therefore, the small reduction in faldaprevir susceptibility conferred by the Q80K polymorphism is likely not sufficient to compromise the response to treatment with faldaprevir and PR.

The impact of the Q80K substitution on *in vitro* susceptibility to simeprevir was more marked than the impact on susceptibility to faldaprevir. Drug sensitivity assays showed that Q80K resulted in mean reductions in simeprevir susceptibility of 9.5-fold and 14.0-fold in genotype 1a and 1b replicon assays, respectively (Fig. 2) (29). It should be noted that since this variant occurs almost exclusively in HCV genotype 1a clinical isolates, it is more relevant to also assess its impact on *in vitro* drug susceptibility in a genotype 1a background. We have not assessed simeprevir susceptibility of clinical isolates encoding Q80K; however, other groups have done so. For example, Bae et al. (12) measured simeprevir susceptibility of 25 genotype 1a clinical isolates from treatment-naïve patients and found two distinct clusters, one comprising 11 Q80K variants with a mean EC_{50} of 20 nM and the other comprising 14 wild-type Q80 isolates with a mean EC_{50} of 3 nM. Importantly, subgroup analyses of clinical trials of simeprevir have shown substantially reduced SVR12 rates in patients with Q80K at baseline (21). Thus, it is likely that patients with HCV genotype 1a will need to be screened for Q80K before treatment with simeprevir (21).

In conclusion, faldaprevir resistance-associated polymorphisms at NS3 R155 and D168 were rarely detected in HCV from untreated patients and do not necessarily compromise the virologic response to faldaprevir-based treatment, although this can be evaluated only on an individual basis. Consistent with previous studies, we found a high prevalence of the Q80K polymorphism in baseline HCV genotype 1a samples; however, the presence of this variant did not compromise the response to faldaprevir-based treatment. Further confirmation of these results is needed in a larger data set where the influence of potentially confounding baseline factors, such as IL28B genotype and degree of cirrhosis, may be assessed. Analysis of data from phase 3 studies, which include a larger sample size of isolates with the Q80K polymorphism, is currently in progress.

ACKNOWLEDGMENTS

We thank the patients, their families, and the clinical investigators. We thank Mithun Ranga and Florian Voss for statistical programming support.

Medical writing assistance, supported financially by Boehringer Ingelheim, was provided by Esther Race (Choice Healthcare Solutions) during the preparation of the manuscript.

REFERENCES

- Ghany MG, Nelson DR, Strader DB, Thomas DL, Seeff LB, American Association for Study of Liver Diseases. 2011. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 54:1433–1444. <http://dx.doi.org/10.1002/hep.24641>.
- Sulkowski MS, Asselah T, Lalezari J, Ferenci P, Fainboim H, Leggett B, Bessone F, Mauss S, Heo J, Datsenko Y, Stern JO, Kulkolj G, Scherer J, Nehmiz G, Steinmann GG, Bocher WO. 2013. Faldaprevir combined with peginterferon alfa-2a and ribavirin in treatment-naïve patients with chronic genotype-1 HCV: SILEN-C1 trial. *Hepatology* 57:2143–2154. <http://dx.doi.org/10.1002/hep.26276>.
- Sulkowski MS, Bourliere M, Bronowicki JP, Asselah T, Pawlotsky JM, Shafraan SD, Pol S, Mauss S, Larrey D, Datsenko Y, Stern JO, Kulkolj G, Scherer J, Nehmiz G, Steinmann GG, Bocher WO. 2013. Faldaprevir combined with peginterferon alfa-2a and ribavirin in chronic HCV genotype-1 patients with prior nonresponse: SILEN-C2 trial. *Hepatology* 57:2155–2163. <http://dx.doi.org/10.1002/hep.26386>.
- Lenz O, Verbinen T, Lin TI, Vijgen L, Cummings MD, Lindberg J, Berke JM, Dehertogh P, Franssen E, Scholliers A, Vermeiren K, Ivens T, Raboisson P, Edlund M, Storm S, Vrang L, de Kock H, Fanning GC, Simmen KA. 2010. *In vitro* resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. *Antimicrob. Agents Chemother.* 54:1878–1887. <http://dx.doi.org/10.1128/AAC.01452-09>.
- Poordad F, Dieterich D. 2012. Treating hepatitis C: current standard of care and emerging direct-acting antiviral agents. *J. Viral Hepat.* 19:449–464. <http://dx.doi.org/10.1111/j.1365-2893.2012.01617.x>.
- Halfon P, Locarnini S. 2011. Hepatitis C virus resistance to protease inhibitors. *J. Hepatol.* 55:192–206. <http://dx.doi.org/10.1016/j.jhep.2011.01.011>.
- Vermehren J, Sarrazin C. 2012. The role of resistance in HCV treatment. *Best Pract. Res. Clin. Gastroenterol.* 26:487–503. <http://dx.doi.org/10.1016/j.bpg.2012.09.011>.
- Vertex Pharmaceuticals Incorporated. 2013. INCIVEK (telaprevir) prescribing information. Vertex Pharmaceuticals Incorporated, Cambridge, MA.
- Merck Sharp & Dohme Corp. 2013. VICTRELIS (boceprevir) prescribing information. Merck Sharp & Dohme Corp., Whitehouse Station, NJ.
- Berger K, Lagacé L, Triki I, Cartier M, Marquis M, Lawetz C, Bethell R, Scherer J, Kulkolj G. 2013. Viral resistance in hepatitis C genotype 1-infected patients receiving the NS3 protease inhibitor faldaprevir (BI 201335) in a phase Ib multiple-rising-dose study. *Antimicrob. Agents Chemother.* 57:4928–4936. <http://dx.doi.org/10.1128/AAC.00822-13>.
- Phenotype Working Group, Drug Development Advisory Group. 2012. Clinically relevant HCV drug resistance mutations figure and tables (updated). *Ann. Forum. Collab. HIV Res.* 14:1–8.
- Bae A, Sun SC, Qi X, Chen X, Ku K, Worth A, Wong KA, Harris J, Miller MD, Mo H. 2010. Susceptibility of treatment-naïve hepatitis C virus (HCV) clinical isolates to HCV protease inhibitors. *Antimicrob. Agents Chemother.* 54:5288–5297. <http://dx.doi.org/10.1128/AAC.00777-10>.
- Paolucci S, Fiorina L, Piralla A, Gulminetti R, Novati S, Barbarini G, Sacchi P, Gatti M, Dossena L, Baldanti F. 2012. Naturally occurring mutations to HCV protease inhibitors in treatment-naïve patients. *Virology* 45:245. <http://dx.doi.org/10.1016/j.virol.2012.09.025>.
- Vicenti I, Rosi A, Saladini F, Meini G, Pippi F, Rossetti B, Sidella L, Di Giambenedetto S, Almi P, De Luca A, Caudai C, Zazzi M. 2012. Naturally occurring hepatitis C virus (HCV) NS3/4A protease inhibitor resistance-related mutations in HCV genotype 1-infected subjects in Italy. *J. Antimicrob. Chemother.* 67:984–987. <http://dx.doi.org/10.1093/jac/dkr581>.
- Cento V, Mirabelli C, Salpini R, Dimonte S, Artese A, Costa G, Mercurio F, Svicher V, Parrotta L, Bertoli A, Ciotti M, Di Paolo D, Sarrecchia C, Andreoni M, Alcaro S, Angelico M, Perno CF, Ceccherini-Silberstein F. 2012. HCV genotypes are differently prone to the development of resistance to linear and macrocyclic protease inhibitors. *PLoS One* 7:e39652. <http://dx.doi.org/10.1371/journal.pone.0039652>.
- Bartels DJ, Sullivan JC, Zhang EZ, Tigges AM, Dorrian JL, De Meyer S, Takemoto D, Dondero E, Kwong AD, Picchio G, Kieffer TL. 2013. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naïve patients prior to treatment. *J. Virol.* 87:1544–1553. <http://dx.doi.org/10.1128/JVI.02294-12>.
- Palanisamy N, Danielsson A, Kulkula C, Yin H, Bondeson K, Wesslen L, Duberg AS, Lennérstrand J. 2013. Implications of baseline polymorphisms for potential resistance to NS3 protease inhibitors in hepatitis C virus genotypes 1a, 2b and 3a. *Antiviral Res.* 99:12–17. <http://dx.doi.org/10.1016/j.antiviral.2013.04.018>.
- Alves R, Queiroz AT, Pessoa MG, da Silva EF, Mazo DF, Carrilho FJ, Carvalho-Filho RJ, de Carvalho IM. 2013. The presence of resistance mutations to protease and polymerase inhibitors in hepatitis C virus sequences from the Los Alamos databank. *J. Viral Hepat.* 20:414–421. <http://dx.doi.org/10.1111/jvh.12051>.
- Zeminian LB, Padovani JL, Corvino SM, Silva GF, Pardini MI, Grotto RM. 2013. Variability and resistance mutations in the hepatitis C virus NS3 protease in patients not treated with protease inhibitors. *Mem. Inst. Oswaldo Cruz* 108:13–17. <http://dx.doi.org/10.1590/S0074-02762013000100002>.
- Leggiewie M, Sreenu VB, Abdelrahman T, Leitch EC, Wilkie GS, Klymenko T, Muir D, Thursz M, Main J, Thomson EC. 2013. Natural NS3 resistance polymorphisms occur frequently prior to treatment in HIV-positive patients with acute hepatitis C. *AIDS* 27:2485–2488. <http://dx.doi.org/10.1097/QAD.0b013e328363b1f9>.
- Janssen Products. 2013. Olysio (simeprevir). Highlights of prescribing information. Janssen Products, LP, Titusville, NJ.
- Manns MP, Bourliere M, Benhamou Y, Pol S, Bonacini M, Trepo C, Wright D, Berg T, Calleja JL, White PW, Stern JO, Steinmann G, Yong C, Kulkolj G, Scherer J, Bocher WO. 2011. Potency, safety, and pharmacokinetics of the NS3/4A protease inhibitor BI201335 in patients with chronic HCV genotype-1 infection. *J. Hepatol.* 54:1114–1122. <http://dx.doi.org/10.1016/j.jhep.2010.08.040>.
- Nishiguchi S, Sakai Y, Kuboki M, Tsunematsu S, Urano Y, Sakamoto W, Tsuda Y, Steinmann G, Omata M. 2014. Safety and efficacy of faldaprevir with pegylated interferon alfa-2a and ribavirin in Japanese patients with chronic genotype-1 hepatitis C infection. *Liver Int.* 34:78–88. <http://dx.doi.org/10.1111/liv.12254>.
- Dieterich D, Asselah T, Guyader D, Berg T, Ceausu E, Preotescu L, Schuchmann M, Mauss S, Ritzu V, Ferenci P, Larrey DG, Maieron A, Biermier M, Stern JO, Scherer J, Kubiak R, Bocher WO. 2011. SILEN-C3: treatment for 12 or 24 weeks with BI201335 combined with peginterferon alfa-2a and ribavirin (P/R) in treatment-naïve patients with chronic genotype-1 HCV infection. *Hepatology* 54:378A.
- Lopez-Labrador FX, Moya A, Gonzalez-Candelas F. 2008. Mapping natural polymorphisms of hepatitis C virus NS3/4A protease and antiviral resistance to inhibitors in worldwide isolates. *Antivir. Ther.* 13:481–494.
- Vallet S, Viron F, Henquell C, Le Guillou-Guillemette H, Lagathu G, Abravanel F, Trimoulet P, Soussan P, Schvoerer E, Rosenberg A, Gouriou S, Colson P, Izopet J, Payan C, ANRS AC11 HCV Group. 2011. NS3 protease polymorphism and natural resistance to protease inhibitors in French patients infected with HCV genotypes 1–5. *Antivir. Ther.* 16:1093–1102. <http://dx.doi.org/10.3851/IMP1900>.
- Pickett BE, Striker R, Lefkowitz EJ. 2011. Evidence for separation of HCV subtype 1a into two distinct clades. *J. Viral Hepat.* 18:608–618. <http://dx.doi.org/10.1111/j.1365-2893.2010.01342.x>.
- De Luca A, Di Giambenedetto S, Prosperi M, Lo Presti A, Torti C, Caudai C, Vicenti I, Saladini F, Almi P, Grima P, Blanc P, Fabbiani M, Rossetti B, Gagliardini R, Ciccozzi M, Zazzi M. 2013. Two distinct HCV genotype 1a clades: geographical distribution and association with natural resistance mutations to HCV NS3/4A inhibitors. *Antiviral Ther.* 18(Suppl 1):A47.
- Lagacé L, White PW, Bousquet C, Dansereau N, Dô F, Llinas-Brunet M, Marquis M, Massariol MJ, Maurice R, Spickler C, Thibeault D, Triki I, Zhao S, Kulkolj G. 2012. *In vitro* resistance profile of the hepatitis C virus NS3 protease inhibitor BI 201335. *Antimicrob. Agents Chemother.* 56:569–572. <http://dx.doi.org/10.1128/AAC.05166-11>.